J Biosci Vol. 43, No. 1, March 2018, pp. 117–126
DOI: 10.1007/s12038-018-9738-5

Glucohexaose-induced protein phosphatase 2C regulates cell redox status of cucumber seedling

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MS received 23 May 2017; accepted 3 November 2017; published online 13 February 2018

Protein Phosphatase 2C (PP2C) is an important phosphatase-like protein in eukaryotic organisms that can negatively regulate protein kinase cascade abscisic acid (ABA) signal system through phosphorylation and carry out vital roles in various cell processes. The previous study indicated that the accumulation of reactive oxygen species (ROS) is a part of mechanism of glucohexaose-induced resistance in cucumber cotyledons, and *CsPP2C80s* might play a crucial role in processes related to ROS produce and signal transduction. To identify the mechanism of *CsPP2C80s* involved in glucohexaose and ABA signaling regulating cell redox status, the effects of glucohexaose and ROS inhibitor pretreatment on endogenous ABA content and ABA signaling genes expression levels of cucumber seedlings were analysed. These results suggest that cucumber *CsPP2C80s* are involved in ROS accumulation and ABA signal transduction pathway induced by glucohexaose, *CsPP2C80s* play a positive regulatory role in process of ABA combined with ABA receptors (PYLs) to activate SNF1-related protein kinases 2 (SnRK2s) and regulate NADPH oxidase to produce extracellular hydrogen peroxide (H_2O_2), providing unequivocal molecular evidence of PP2C-mediated ABA response mechanisms functioning in cell redox status induced by glucohexaose.

Keywords. ABA; glucohexaose; PP2C; qRT-PCR; ROS inhibitor

1. Introduction

In the process of growth and development, the plants form the ability to resist pests and diseases. Induced resistance (IR) is a special acquired immunity after plant is stimulated by biological or non-biological factors. Compounds such as oligosaccharides and proteins can induce the plant to produce defensive reactions (Strobel *et al.* 1996; Thuerig *et al.* 2006; Saravanakumar *et al.* 2007; Huang *et al.* 2012). Oligonucleotides are the elicitors that are earliest studied (Sharp *et al.* 1984) Glucohexaose, synthesized by Research Center for Eco-Environmental Science, Chinese Academy of Science as an oligosaccharide. **U**ronic acid oligosaccharide of plant cell wall derives regulatory factors, which can stimulate the expression of defense genes in pathogen infection process, and enhances pp34 protein phosphorylation (Reymond *et al.* 1995). We found that cucumber resistance systems were stimulated and acquired defensive capacity to *Pseudoperonospora cubensis* after glucohexaose incubation (Hao *et al.* 2013). These oligosaccharides induce plant defense systems as signaling molecules.

Protein Phosphatase 2C (PP2C) is evolutionary conserved from prokaryotes to eukaryotes and play a prominent role in various signaling pathways and plant development. In plants, PP2Cs are considered to be the largest protein phosphatase family as regulators of signal transduction pathways and involved in plant development. Studies have showed that protein dephosphorylation plays a crucial role in regulating signaling cascades in response to abscisic acid (ABA) and hydrogen peroxide (H_2O_2) in guard cells (Schmidt *et al.* 1995; Mori and Muto 1997). PP2C is a direct target of reactive oxygen species (ROS) in ABA signaling (Meinhard and Grill 2001; Meinhard *et al.* 2002). The signal pathways most closely related to ROS accumulation includes mitogen-activated protein kinase (MAPK), ABA and Ca^{2+} signal. ROS is participates in the process of plant growth,

development, hormonal signaling and responses to environmental stimuli in plants (Apel and Hirt 2004; Mittler *et al.* 2004; Kwak *et al.* 2006; Torres *et al.* 2006; Suzuki *et al.* 2011). Currently, the physiological and biochemical changes is associated with the outbreak of ROS, the production of plant hormone, the pathogenesis-related proteins and the defensive enzymes (Liu *et al.* 2004; Chisholm *et al.* 2006; Vos *et al.* 2013). Coincidentally, PP2C plays a critical role in these signals (Bertauche *et al.* 1996; Gaits *et al.* 1997; Yoshida *et al.* 2006). At present, six kinds of PP2C protein phosphatases have been found in plants, including ABI1, ABI2, HAB1, HAB2, AHG1 and PP2CA/AHG3. Genetic research has indicated that PP2Cs, ABI1 and ABI2 of Arabidopsis were as negative regulators participating in ABA signaling pathway (Armstrong *et al.* 1995; Sheen 1998; Gosti *et al.* 1999; Merlot *et al.* 2001). At present, a large number of *PP2C* genes have been cloned in plants, and the studies focus on their roles in different signaling pathways, for example, the biological function of ABI1 and ABI2 has been understood more clearly (Leung *et al.* 1994). Phosphorylation of PP2C protein plays an important role in the ABA signaling pathway, and some PP2C protein kinases are isolated and described as ABA signaling factors (Hirayama and Shinozaki 2010). *AtPP2C* participates in stomatal closure and plays a negative regulatory role in ABA signaling (Tahtiharju and Palva 2001). *MpABI1* is a negative regulator of ABA signaling, which provide molecular evidence of PP2C-mediated ABA response mechanism function (Tougane *et al.* 2010). Screening mutants and yeast two-hybrid methods were used to determine a new ABA receptor PYR / PYL / RCAR protein in Arabidopsis (Park *et al.* 2009; Ma *et al.* 2009). The increase of residues in the ABA/PYL10 complex may enhance the interactions between PP2C and PYL10, leading to an increase in PP2C phosphatase inhibition by PYL10 (Li *et al.* 2015). In normal growing conditions, dephosphorylation of clade A PP2C makes SnRK2 inactivation and ABA signal keeping silent. Once the environmental conditions or developmental signals stimulate plants, ABA will be induced and combine with PYR / PYL / RCAR interacting with the clade A of PP2C, inhibiting the activity of SnRK2. As SnRK2 phosphorylate downstream transcription factors or membrane protein factors, ABA signal pathway is open (Raghavendra *et al.* 2010). ABA can induce ROS accumulation and stomatal closure but NADPH oxidase double mutant *atrbohD/F* cannot (Kwak *et al.* 2003). ABA can elevate H_2O_2 content in corn embryo and seedlings (Guan *et al.* 2000; Jiang and Zhang 2002; Pei *et al.* 2000; Zhang *et al.* 2001). The activities of ABI1 and ABI2 are suppressed by H_2O_2 in Arabidopsis (Meinhard and Grill 2001, 2002).

Our previous study found that the content of O^{2-} and H_2O_2 dramatically increased after 5h by glucohexaose treatment in cucumber, and then O^{2-} gradually reduced while H_2O_2 increased sustainable to 10h after treatment, however, the accumulation of ROS inhibited by diphenyleneiodonium chloride (DPI, an inhibitor of NADPH oxidase) and dimethylthiourea (DMTU, a ROS scavenger) pretreatment. Two-dimensional electrophoresis (2-DE) coupled with mass spectrometry analysis revealed that AtPP2C 80 (15240071) related to the outbreak of ROS activated in the control of redox state by glucohexaose. Multiple sequence alignment analysis indicated that two PP2C80 related proteins (Cucsa201270 and Cucsa342170) were identity with AtPP2C 80 in cucumber. The results indicated that PP2C was a ROS-related and glucohexaose-induced protein, which might have important function either in ROS accumulation and signal transduction (Hao *et al.* 2014).

2. Materials and methods

2.1 *Materials and experimental design*

Cucumber seeds (Jinyan No.4) were soaked in distilled water for 2 h, sterilized with 75 % ethanol for 60 s, 2.5 % NaClO for 13 min and then washing at least three times. The seeds were placed on soaked gauze and germinate at 25–28°C. Until the cotyledons expanded, the seedlings were used for subsequent experiments.

To investigate the effect of glucohexaose in the process of *CsPP2C80s* expression and ABA content changes, a part of the seedlings were sprayed with 50 $\mu g.mL^{-1}$ glucohexaose and deionized water was used to spray control seedlings. Seedlings were sampled at 0, 1, 5, 9, 10 and 14 h after glucohexaose treatment, stored at -80°C.

To investigate the effect of DPI (an inhibitor of NADPH oxidase) and DMTU in the process of the oxidative burst, the materials were divided into six groups. The first (control plants) and second groups of materials were sprayed with deionized water and 50 $\mu g.mL^{-1}$ glucohexaose respectively after incubating with deionized water in advance for 4 h. The third and fourth groups of materials were sprayed with 50 $\mu g.mL^{-1}$ glucohexaose after incubating with 100μM DPI and 5mM DMTU for 4 h respectively. The fifth and sixth groups of materials were sprayed with deionized water after incubating with 100μM DPI and 5mM DMTU for 4 h respectively. Seedlings were harvested at 5 h after being sprayed by glucohexaose or deionized water, and then stored at −80°C.

2.2 *To determine ABA concentrations*

Approximately 0.5g samples were collected into liquid nitrogen and homogenized in 80% chilled methanol. The supernatant was collected after centrifugation, purified through a C18 cartridge column and eluted with 80% methanol. The ABA level was determined using the enzyme-

linked immunosorbent assay (ELISA) test kit according to the instructions.

2.3 *Quantitative real-time PCR (qRT-PCR) expression analysis*

Total RNA was isolated from different samples of cucumber seedlings sprayed by glucohexaose, glucohexaose with DPI pretreatment, glucohexaose with DMTU pretreatment. The cDNA was generated using a QuantScript RT kit (TianGen). The TianGen SuperReal PreMix Plus (SYBR Green) PCR kit was used for qRT-PCR analysis. All primer sequences were showed in table 1.

Quantitative real-time PCR analysis was conducted using a Roche LightCycler 480. Relative quantification of the gene expression was calculated by the $2^{-\triangle\triangle Ct}$ method. All experiments were based on three biological replicates.

3. Results

3.1 *Changes of PP2C after the treatment of glucohexaose*

There are about 60 PP2C genes in cucumber. Our previous study showed that AtPP2C 80 was a glucohexaose-induced protein, which might have function in ROS accumulation and signal transduction. Multiple sequence alignment analysis indicated that two CsPP2C80s are identity with AtPP2C 80 in cucumber. In order to detect the relationship between *CsPP2C80s* and genes involved in ABA signaling from other plants, nucleotide sequences from Arabidopsis (*ABI1, ABI2, HAB1, HAB2, AtPP2CA, AthPP2C5 and AtPP2C80*), beeches (*FsPP2C1, FsPP2C2*), alfalfa (*MP2C*), corn (*ZmPP2C*), strawberries (*FaABI1*), and cucumber (*CsPP2C80–1, CsPP2C80–2*) were aligned using Molecular Evolutionary Genetics Analysis (MEGA) software. The phylogenetic tree indicated that *CsPP2C80–1* was the highest homology with strawberries *FaABI1* and *CsPP2C80–2* was the highest homology with beech FsPP2C2 (figure 1).

In our previous study, to clarify the role and mechanism of ROS burst out in the resistance of cucumber induced by glucohexaos, the differential proteomics method was used to study the oligodeoxynucleotides elicitor and the effect of NADPH oxidase inhibitor (DPI) and H_2O_2 scavenger (DMTU) on the different expression of protein in cucumber cotyledons. Studies have shown that PP2C protein content is significantly upregulated after glucoside induction, whereas DMTU and DPI pretreatment will inhibit this increase in some extent, and DMTU inhibitory effect is more obvious (Hao et al. 2014).

To indicate whether *CsPP2C80s* were involved in glucohexaose-induced response, the mRNA expression pattern of *CsPP2C80s* under glucohexaose-induced treatment was examined by qRT-PCR (figure 2A, B). The transcripts of *CsPP2C80s* were significantly induced in response to glucohexaose treatment. The expression levels of *CsPP2C80s* were increased rapidly from 0 h to 9 h peaked at 9 h, and then decreased continuously.

Table 1. Primer sequences used for qRT-PCR

Gene name	Primer sequence (5′ to 3′)
18S Rrna-F	ATGATAACTCGACGGATCGC
18S Rrna-R	CTTGGATGTGGTAGCCGT
CsPP2C80–1-F	ATGTAGTTGGCGTTGCGGATGGT
CsPP2C80–1-R	TAGCCGTAGTGCCGGAGTGAGCT
CsPP2C80–2-F	TTTTCTATTCCCTCCGTGTC
CsPP2C80–2-R	CCATTATTGTTAGGCTGCTC
PYL1-F	CTTTGTGGAAGCCTTGATT
PYL1-R	TATACCCTCCTGCCTGTCC
PYL2-F	CAGGCTTAGGAACTACTCT
PYL2-R	TAAATGACTCAACCACCAG
PYL3-F	GCTACACCGCCACTACTCCC
PYL3-R	GCAGACCTGACACCACCTCC
SnRK2.1-F	CGGCATACATAGCACCAG
SnRK2.1-R	GCACCGACCAACATAACA
SnRK2.2-F	CAGAAATCAAGAAGCACCCA
SnRK2.2-R	CTCCAAGGCACAAACAAAGT
Rboh F-F	CCAGCACAGGACTACCAT
Rboh F-R	AGGAGCGTGACAATCTTT
Rboh A1-F	GGAGGAGTTTGTGGAGGTG
Rboh A1-R	CGTCGATATTGATTATAGTGGC
Rboh A2-F	GAGCCAGAACATACAGGGAC
Rboh A2-R	ATGGAGAATGACGCCAACTA

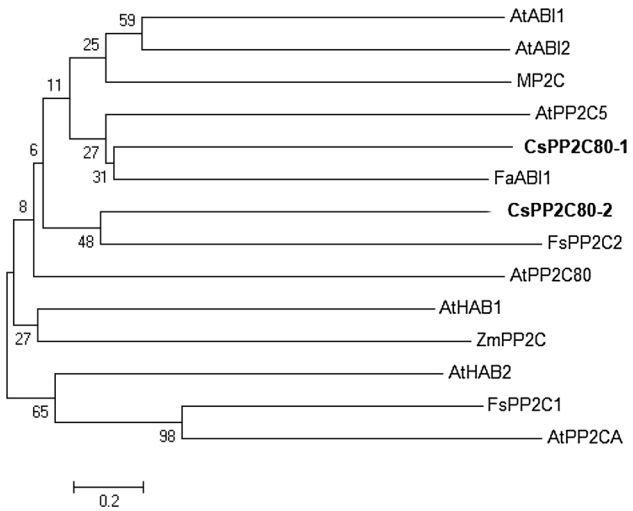

Figure 1. Phylogenetic tree analysis of different sources of plant PP2Cs. The tree was constructed using the neighbor-joining method provided in MEGA 4.0 software. Bootsrap values (1,000 replicates) were given below the branches.

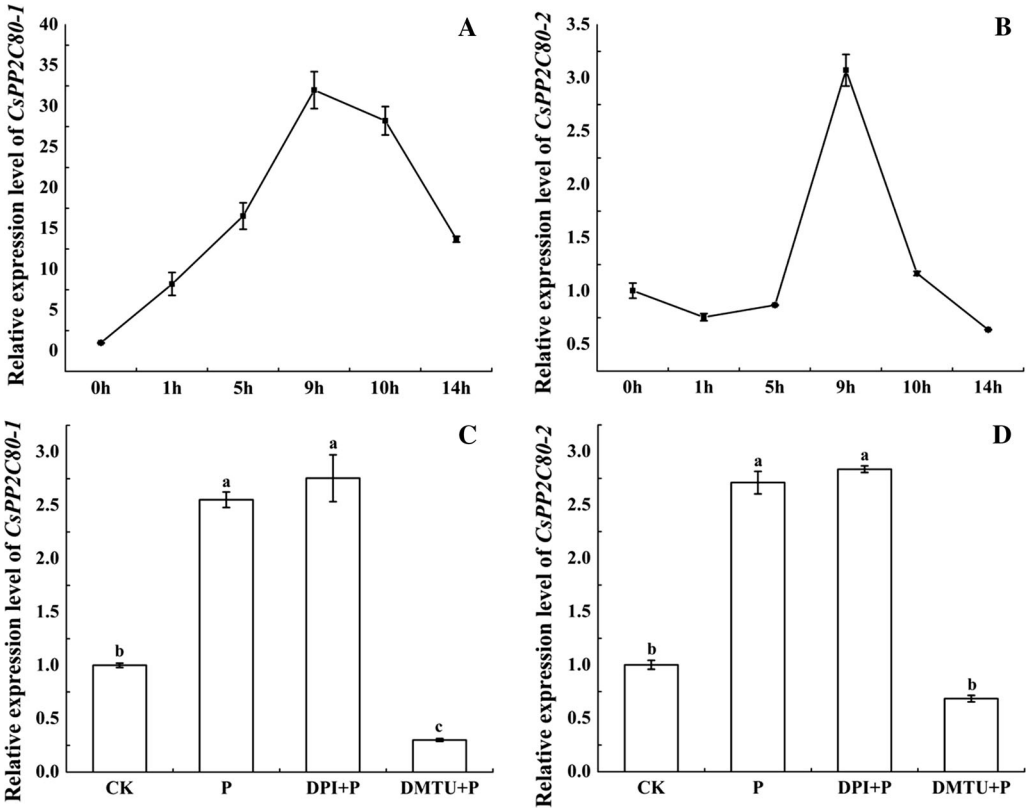

Figure 2. Relative expression patterns of *CsPP2C80s* mRNA. (**A** and **B**) Treated with glucohexaose. (**C** and **D**) Treated with P, DPI+ P and DMTU+P respectively. Each sample was individually assayed in triplicate. CK: Control check. Sterile water treatment; P: Glucohexaose treatment 5 h; DPI+P/DMTU+P: DPI/DMTU pretreatment 4h and glucohexaose treatment 5h; DPI/DMTU: DPI/DMTU treatment 4 h. Lowercase letters: $P<0.05$.

ROS (H_2O_2 and O^{2-}) significant accumulation occurred at 5h after treat with glucohexaose. DMTU and DPI pretreatment suppressed the effect of glucohexaose (Hao *et al.* 2014). To evaluate whether *CsPP2C80s* expression were affected by ROS inhibitors, the levels of *CsPP2C80s* transcript in seedlings were studied by qRT-PCR analysis (figure 2C, D). Following DMTU pretreatment, the expression of *CsPP2C80s* decreased significantly compared to glucohexaose treatment. The inhibiting effect was not obvious after DPI treatment.

3.2 *Alteration of ROX status induced by glucohexaose affected ABA levels and ABA-responsive gene transcripts*

To confirm the mechanism of *CsPP2C80s* in the regulation of cucumber seedling redox status induced by glucohexaose, several inducing-related physiological parameters were measured, including ABA content and a set of ABA-responsive genes, such as *PYL*, *SnRK2*, *RbohA*.

The results showed that ABA content increased continuously from 0 h to 9 h after glucohexaose treatment, the content of ABA were extremely high at 9 h, and then declined rapidly. ABA content returned to the control treatment level at 14 h (figure 3A). The exaltation of ABA was later than the accumulation of ROS after glucohexaose treatment. Meanwhile, the ABA content failed to accumulate after DPI and DMTU pretreatment (figure 3B).

Previous studies have shown that PYR/PYL/RCAR could combine with ABA directly, and different family member of PYR/PYL/RCAR selected different ABA steric configuration (Szostkirwiez *et al.* 2010). PYR/PYL/RCAR receptors could inhibit PP2C phosphatase activity through interaction with clade A of PP2C.

The qRT-PCR analysis indicated that the family members of ABA receptors were differentially expressed in response to various treatments. The expression patterns of *PYL1*, *PYL2*, and *PYL3* were significantly up-regulated and peaked at 10h, 9 h and 10 h after glucohexaose treatment, respectively (figure 4A, B, C). However, the expression levels of *PYL1*, *PYL2* and *PYL3* were all markedly down-regulated after DPI and DMTU pretreatment compared to glucohexaose treatment alone (figure 4D, E, F). Meanwhile, *PYL1* and *PYL3* expression level was obviously induced by glucohexaose treatment, but the effect of DPI and DMTU pretreatment on *PYL1* expression was more obvious.

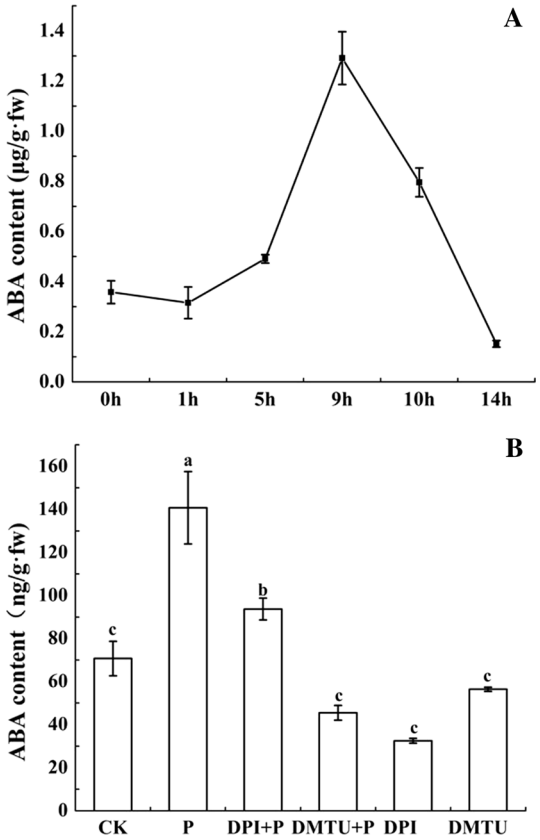

Figure 3. The content of endogenous ABA. (**A**) Treated with glucohexaose. (**B**) Treated with P, DPI +P, DMTU+P, DPI and DMTU respectively. Each sample was individually assayed in triplicate. CK: Control check. Sterile water treatment; P: Glucohexaose treatment 5 h; DPI+P/DMTU+P: DPI/DMTU pretreatment 4h and glucohexaose treatment 5h; DPI/DMTU: DPI/DMTU treatment 4 h. Lowercase letters: $P<0.05$.

In plants, sucrose nonfermenting-1 (SNF1)-related protein kinases (SnRKs) involve in the stress responses and metabolic regulation (Halford and Hey, 2009; Hey *et al.* 2010). SnRKs can be subdivided into three subfamilies: SnRK1, SnRK2, and SnRK3 (Halford, 2005). A key role for SnRK2 in ABA signaling pathway has been found with the identification of PYR/PYL/RCAR proteins as ABA receptors and involve in ABA signalling (Leung *et al.* 1997). ABA regulates the activity of SnRK2, which, in turn, activates ABA response element binding proteins (AREBPs) inducing gene expression (Coello *et al.* 2011). In this experiment, our qRT-PCR analysis indicated that *SnRK2.1* and *SnRK2.2* expression levels were induced by glucohexaose treatment, and the highest expression level were observed at 9h and 10h, respectively (figure 5A, B). However, the expression pattern of *SnRK2.1* and *SnRK2.2* were significantly inhibited by pretreatment with DPI and DMTU (figure 5C, D).

A key factor in the network of ROS produce is the special respiratory burst or NADPH oxidase (Torres and Dangl 2005). NADPH oxidases are the source of ROS produce in ABA signaling and other processes, such as plant cell growth and defense response (Torres *et al.* 2002; Foreman *et al.* 2003; Kwak *et al.* 2006). RbohA and RbohB are involved in H_2O_2 accumulation and viral resistance in *Nicotiana benthamiana* (Yoshioka *et al.* 2003). RbohD and RbohF are required for ROS accumulation intermediates in plant defense response and participate in ROS-dependent ABA signaling (Torres *et al.* 2002; Foreman *et al.* 2003; Bright *et al.* 2006). In this experiment, the qRT-PCR analysis indicated that *RbohA1*, *RbohA2* and *RbohF* were induced in response to glucohexaose treatment. The transcript pattern of *RbohA1*, *RbohA2* and *RbohF* increased at early stage, decreased from 2 h to 9 h, and then increased again (figure 6A, B, C). The results suggested that glucohexaose treatment could induce *Rbohs* gene expression at early stage. The expression pattern of *RbohA1* and *RbohF* were inhibited by pretreatment with DPI and DMTU (figure 6D, F), but the expression of *RbohA2* was further up-regulated by pretreatment with DPI and DMTU (figure 6E).

4. Discussion

In agricultural production, how to prevent disease without pesticide residues has attracted more attention. Elicitors can protect crops from pest, disease or environmental stress. Researches show that elicitors can cause ROS accumulation rapidly and trigger defense response (Bradley *et al.* 1992; Svalheim and Robertsen 1993; Nürnberger *et al.* 1994; Auh and Murphy 1995). Glucohexaose is a synthetic oligosaccharide elicitor that can activate plants acquired resistance to *Pseudoperonospora cubensis* (Fan *et al.* 2003). Our previous study confirmed that the ROS accumulation was a part of the mechanisms of glucohexaose-induced resistance and PP2C might play a crucial role in processes related to ROS produce and signal transduction (Hao *et al.* 2014). We had no idea whether glucohexaose-induced resistance was related to ABA signaling, why PP2C accumulated after ROS content increased and whether CsPP2C80s were involved in ABA signaling pathway. Therefore, we sought to further investigate the possible link among ROS accumulation, PP2C, ABA signaling and glucohexaose-induced resistance. We reported that glucohexaose-induced resistance was related to ROS and ABA signaling, that *CsPP2C80s* played an important role in process of ABA combined with PYLs to activate SnRK2s and thereby regulated NADPH oxidase to produce extracellular H_2O_2 in cucumber. These results provided important clues of the mechanism of glucohexaose-induced resistance. We found that the expression of *CsPP2C80s* was induced after glucohexaose treatment, and this increase would be inhibited by active oxygen inhibitors

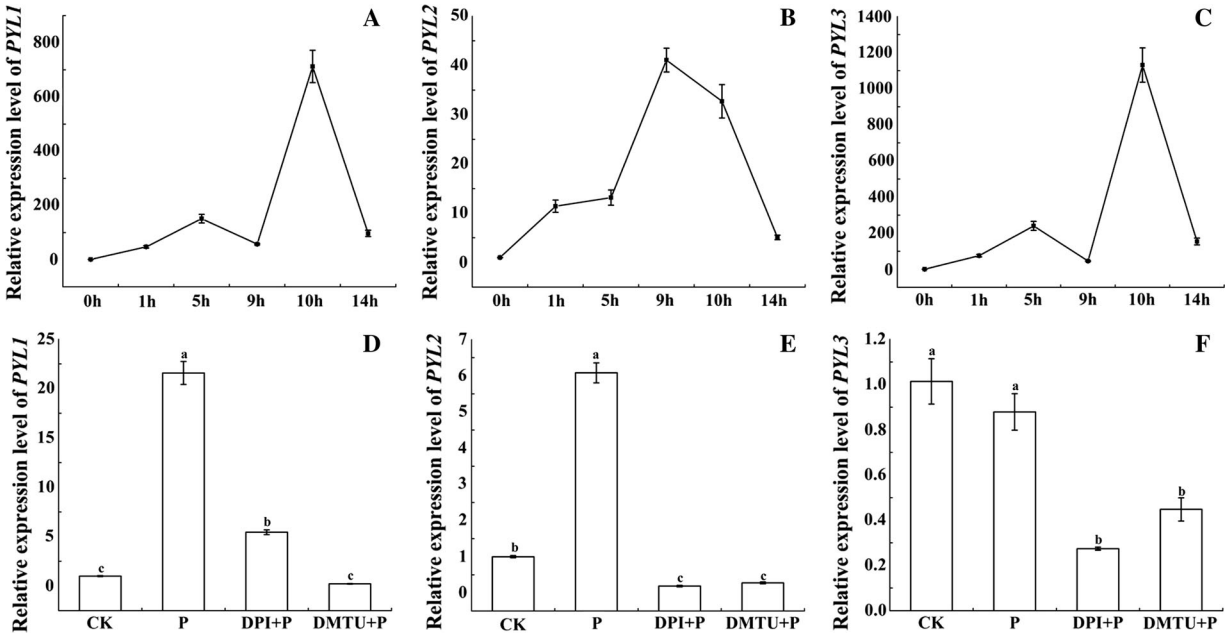

Figure 4. Relative expression patterns of *PYLs* mRNA using the qRT-PCR method. (**A**) Relative expression pattern of *PYL1* mRNA after glucohexaose treatment; (**B**) *PYL2*; (**C**) *PYL3*; (**D**) Relative expression patterns of *PYL1* gene after P, DPI+ P and DMTU+P treatments, respectively; (**E**) *PYL2*; (**F**) *PYL3*. Each sample was individually assayed in triplicate. CK: Control check. Sterile water treatment; P: Glucohexaose treatment 5 h; DPI+P/DMTU+P: DPI/DMTU pretreatment 4h and glucohexaose treatment 5h. Lowercase letters: $P<0.05$.

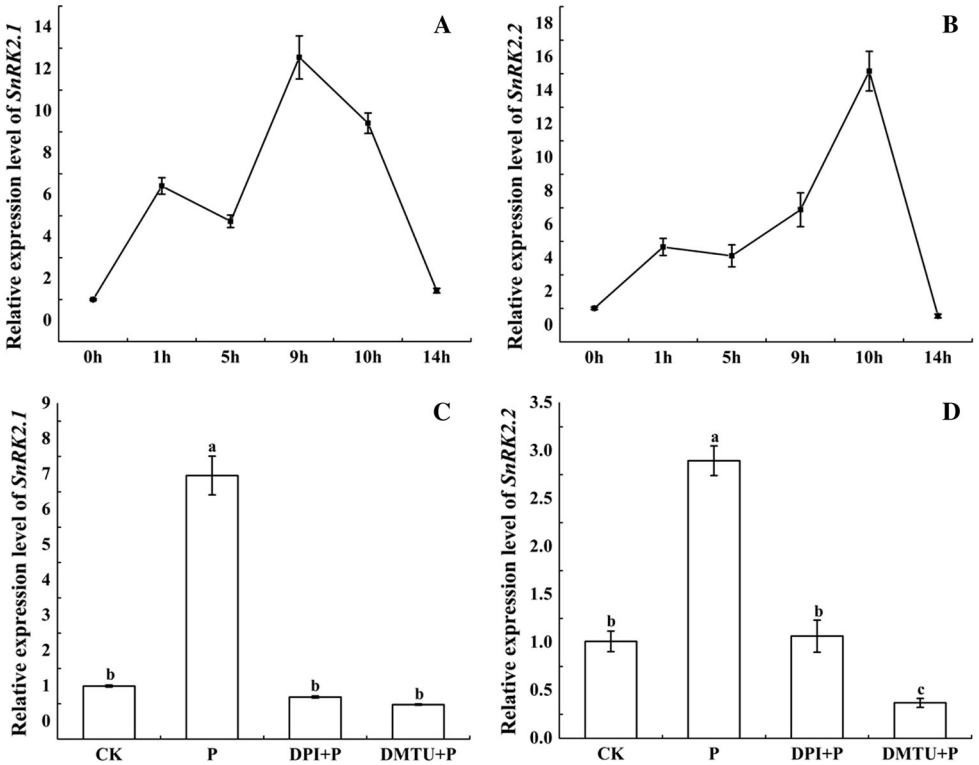

Figure 5. Relative expression patterns of *SnRK2s* mRNA using the qRT-PCR method. (**A**) *SnRK2.1* gene expression pattern after glucohexaose treatment; (**B**) *SnRK2.2*; (**C**) *SnRK2.1* gene expression patterns after P, DPI+ P and DMTU+P treatments, respectively; (**D**) *SnRK2.2*. Each sample was individually assayed in triplicate. CK: Control check. Sterile water treatment; P: Glucohexaose treatment 5 h; DPI+P/DMTU+P: DPI/DMTU pretreatment 4h and glucohexaose treatment 5h. Lowercase letters: $P<0.05$.

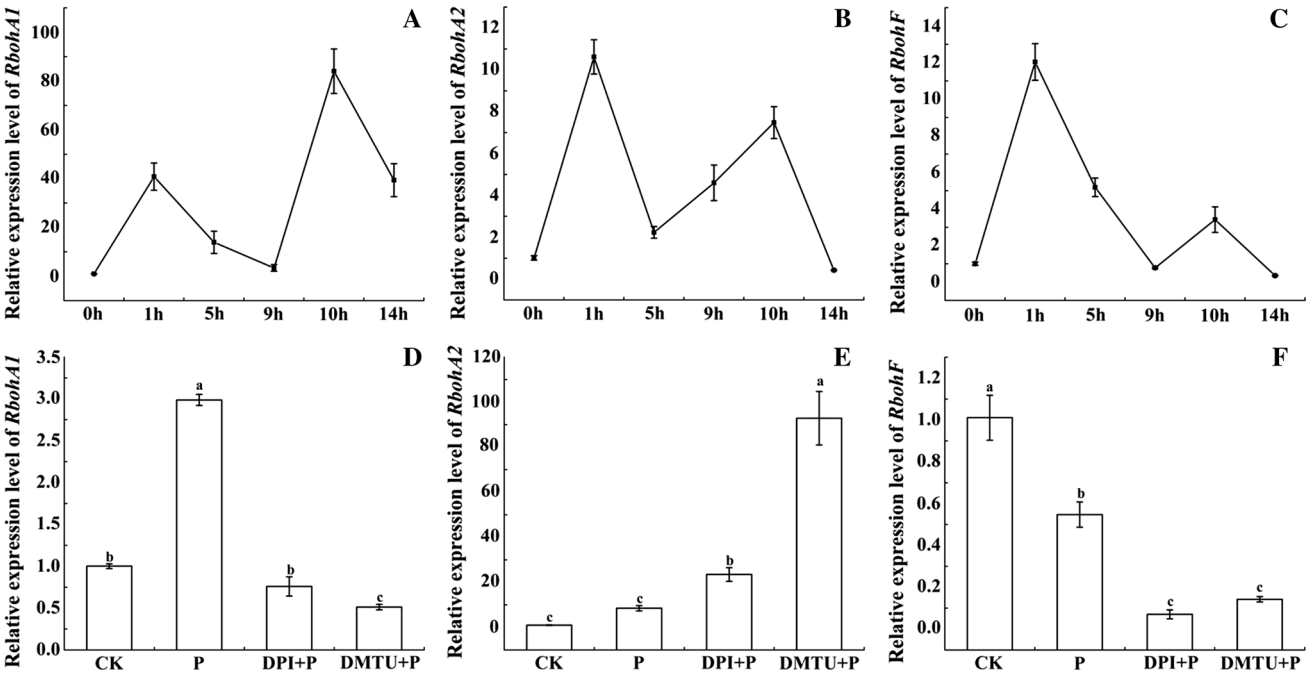

Figure 6. Relative expression patterns of *Rbohs* mRNA using the qRT-PCR method. (**A**) *RbohA1* gene expression pattern after glucohexaose treatment; (**B**) *RbohA2*; (**C**) *RbohF*; (**D**) *RbohA1* gene expression patterns after P, DPI+ P and DMTU+P treatments, respectively; (**E**) *RbohA2*; (**F**) *RbohF*. Each sample was individually assayed in triplicate. CK: Control check. Sterile water treatment; P: Glucohexaose treatment 5 h; DPI+P/DMTU+P: DPI/DMTU pretreatment 4h and glucohexaose treatment 5h. Lowercase letters: $P<0.05$.

DMTU and DPI pretreatment, which indicated that CsPP2C80s might participate in the accumulation of ROS by glucohexaose regulation in cucumber seedlings.

ABA signaling pathway includes PYR/PYL/RCAR, PP2C, and SnRK2. PP2Cs exclusive interact with ABA receptors and SnRK2s (Umezawa *et al.* 2009; Ma *et al.* 2009; Park *et al.* 2009). The diversity of PP2C in plants shows the diversity of signal transduction mechanisms in different tissues and organs. PP2C homolog gene *PeHAB1* is a new negative regulator of ABA signaling in poplar (Chen *et al.* 2015). Previous reports found that clade A of PP2C inhibited the release of signaling triggered by ABA (Fuchs *et al.* 2013; Tougane *et al.* 2010). Beech *FsPP2C1* is reported to be a positive regulator in ABA signaling pathway, and involved in the synthesis and metabolism of gibberellic acid (GA) (Reyes *et al.* 2006). *FaABI1* had been detected to be a negative regulator of strawberry fruit ripening, and it was suggested that the ABA-FaPYR1-FaABI1-FaSnRK2 signalling pathway might be an important mechanism in non-climacteric fruit (Jia *et al.* 2013). In this study, the phylogenetic tree indicated that *CsPP2C80–1* was the highest homology with strawberries *FaABI1* and *CsPP2C80–2* was the highest homology with beech FsPP2C2 (figure 1). Furthermore, CsPP2C80s had close genetic relationship with Arabidopsis class K of PP2C. The biological function of PP2C K class is still rarely reported.

In our previous study, the content of H_2O_2 and superoxide anion dramatically accumulated after 5h and the expression of PP2C was induced by glucohexaose treatment in cucumber, however, the accumulation of ROS inhibited by DPI and DMTU pretreatment. With the question of why PP2C induced after ROS content increased, whether glucohexaose-induced resistance was related to ABA signaling and whether the PP2C was involved in ABA signaling pathway. We sought to further investigate the possible link among ROS accumulation, CsPP2C80s, ABA signaling and glucohexaose-induced resistance.

In this work, the endogenous ABA content increased obviously at 9 h after glucohexaose treatment, but DMTU and DPI pretreatment obviously inhibited the effect. ABA content increased slightly later than the accumulation of ROS. ROS accumulation and ABA content increased induced by glucohexaose. The relative expression levels of *CsPP2C80s* also induced by glucohexaose, reached the highest expression at 9 h, and DMTU, DPI pretreatment inhibited such exaltation in a certain extent. The changes of CsPP2C80s were consistent with ROS accumulation. Meanwhile, the relative expression of these allele genes of *PYL1* and *PYL2*, *SnRK2.1* and *SnRK2.2* also increased significantly after glucohexaose treatment, and DMTU, DPI pretreatment abolished this increase too. Endogenous ABA accumulation caused the changes of NADPH oxidase *Rbohs* gene expression at early stage, whereas DMTU or DPI

pretreatment restrained these changes. Thus, CsPP2C80s were involved in process of ROS produce and signal transduction induced by glucohexaose. We speculated that glucohexaose induce resistance partially due to ROS accumulation opening ABA signaling and *CsPP2C80s* might play a positive regulatory role in process of ABA combined with PYLs to activate SnRK2s and regulated NADPH oxidase to produce extracellular H_2O_2 in cucumber. These results provided important clues of the mechanism of glucohexaose-induced resistance and lay the foundation of further evidence of reverse genetics to demonstrate the specific role of PP2C in cucumber.

Acknowledgements

This work was supported by the Program for Excellent Researcher in University of Liaoning Province (LR2014019) and the Talents Engineering of Liaoning Province Project (2014921040). All the authors are thankful to Prof. J Ning for providing glucohexaose.

References

Apel K and Hirt H 2004 Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol*. **55** 373–399

Armstrong F, Leung J, Grabov A, Brearley J, Giraudat J and Blatt MR 1995 Sensitivity to abscisic acid of guard-cell K+ channels is suppressed by abi1–1, a mutant Arabidopsis gene encoding a putative protein phosphatase. *Proc. Natl. Acad. Sci. USA* **92** 9520–9524

Auh CK and Murphy TM 1995 Plasma membrane redox enzyme is involved in the synthesis of O_2^- and H_2O_2 by Phytophthoraelicitor-stimulated rose cells. *Plant Physiol*. **107** 1241–1247

Bertauche N, Leunq J and Giraudat J 1996 Protein phosphatase activity of abscisic acid insensitive 1 (ABI1) protein from *Arabidopsis thaliana*. *Eur. J. Biochem*. **241** 193–200

Bradley DJ, Kjellbom P and Lamb CJ 1992 Elicitor-and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Cell* **70** 21–30

Bright J, Desikan R, Hancock JT, Weir IS and Neill SJ 2006 ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H_2O_2 synthesis. *Plant J*. **45** 113–122

Chen J, Zhang D, Zhang C, Xia X, Yin W and Tian Q 2015 A putative PP2C-encoding gene negatively regulates ABA signaling in Populus euphratica. *PLoS ONE* **10**(10) e0139466

Chisholm ST, Coaker G, Day B and Staskawicz BJ 2006 Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* **124** 803–814

Coello P, Hey SJ and Halford NG 2011 The sucrose non-fermenting-1-related (SnRK) family of protein kinases: potential for manipulation to improve stress tolerance and increase yield. *J. Exp. Bot*. **62** 883–893

Fan HY, Bao-Ju LI, Chun-Mao L, Tian-Lai LI and Zhou BL 2003 Study on cucumber plant resistance introduced by glucohexaose against pseudoperonospora cubensis disease. *Plant Prot.* (*China*) **1** 14–16

Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, *et al*. 2003 Reactive oxygen species produced by nadph oxidase regulate plant cell growth. *Nature* **422** 442–446

Fuchs S, Grill E, Meskiene I and Schweighofer A 2013 Type 2c protein phosphatases in plants. *FEBS J*. **280** 681–693

Gaits F, Shiozaki K and Russell P 1997 Protein phosphatase 2C acts independently of stress-activated kinase cascade to regulate the stress response in fission yeast. *J. Biol. Chem*. **272** 17873–17879

Gosti F, Beaudoin N, Serizet C, Webb AA, Vartanian N and Giraudat J 1999 ABI1 protein phosphatase 2C is a negative regulator of abscisic acid signaling. *Plant Cell* **11** 1897–1910

Guan Z, Prado A, Melzig J, Heisenberg M, Nash HA and Raabe T 2000 Mushroom body defect, a gene involved in the control of neuroblast proliferation in drosophila, encodes a coiled–coil protein. *Proc. Natl. Acad. Sci. USA* **97** 8122–8127

Halford NG and Hey SJ 2009 Snf1-related protein kinases (SnRKs) act within an intricate network that links metabolic and stress signalling in plants. *Biochem. J.* **419** 247–259

Halford NG 2005 Regulation of carbon and amino acid metabolism: roles of sucrose nonfermenting-1-related prote in kinase-1 and general control nonderepressible-2-related protein kinase. *Adv. Bot. Res*. **43** 93–142

Hao Y, Wu C, Zhao D, Thung L, Yu Y and Fan H 2013 Proteomic analysis of glucohexaose induced resistance to downy mildew in *Cucumis sativus*. *Aust. J. Crop Sci.* **7** 1242–1251

Hao Y, Lin C, Fan H, Yu Y, Li N and Chen S 2014 Proteomic analysis of *Cucumis sativus* cotyledons after glucohexaose treatment as a part of ROS accumulation related resistance mechanism. *Proteome Sci*. **12** 34–44

Hey SJ, Byrne E and Halford NG 2010 The interface between metabolic and stress signaling. *Ann. Bot.* **105** 197–203

Hirayama T and Shinozaki K 2010 Research on plant abiotic stress responses in the post-genome era: past, present and future. *Plant J*. **61** 1041–1052

Huang CH, Vallad GE, Zhang S, Wen A, Balogh B, Figueiredo JFL, Behlau F, Jones JB, *et al*. 2012 Effect of application frequency and reduced rates of acibenzolar-S-methyl on the field efficacy of induced resistance against bacterial spot on tomato. *Plant Disease* **96** 221–227

Jiang MY and Zhang JH 2002 Role of abscissic acid in water stress-induced antioxidant defense in leaves of maize seedlings. *Free Radic. Res.* **36** 1001–1015

Jia HF, Lu D, Sun JH, Li CL, Xing Y, Qin L and Shen YY 2013 Type 2C protein phosphatase ABI1 is a negative regulator of strawberry fruit ripening. *J. Exp. Bot.* **64** 1677–1687

Kwak JM, Mori IC, Pei ZM, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Bodde S, *et al*. 2003 Nadph oxidase atrbohd and atrbohf genes function in ros-dependent aba signaling in Arabidopsis. *The EMBO J.* **22** 2623–2633

Kwak JM, Nguyen V and Schroeder JI 2006 The role of reactive oxygen species in hormonal responses. *Plant Physiol*. **141** 323–329

Leung J, Bouvier-Durand M, Morris PC, Guerrier D, Chefdor F and Giraudat J 1994 Arabidopsis ABA response gene abi1: features of a calcium-modulated protein phosphatase. *Science* **264** 1448–1452

Leung J, Merlot S and Giraudat J 1997 The Arabidopsis ABSCISIC ACID-INSENSITIVE2 (*ABI2*) and *ABI1* genes encode homologous protein phophatases 2C involved in abscisic acid signal transduction. *The Plant Cell* **9** 759–771

Li J, Shi C, Sun D, He Y, Lai C, Lv P, Xiong Y, Zhang L, *et al*. 2015 The HAB1 PP2C is inhibited by ABA-dependent PYL10 interaction. *Sci*. *rep*. **5** 10890

Liu H, Zhu Z and Lv G 2004 Effect of chilling tolerance and active oxygen scavenging system of grafted watermelon stress on low temperature. *Appl. Ecol. (China)* **15** 659–662

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A and Grill E 2009 Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* **324** 1064–1068

Meinhard M and Grill E 2001 Hydrogen peroxide is a regulator of ABI1, a protein phosphatase 2C from Arabidopsis. *FEBS Lett.* **508** 443–446

Meinhard M, Rodriguez PL and Grill E 2002 The sensitivity of ABI2 to hydrogen peroxide links the abscisic acid-response regulator to redox signaling. *Planta* **214** 775–782

Merlot S, Gosti F, Guerrier D, Vavasseur A and Giraudat J 2001 The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. *Plant J.* **25** 295–303

Mittler R, Vanderauwera S, Gollery M and Van Breusegem F 2004 Reactive oxygen gene network of plants. *Trends Plant Sci.* **9** 490–498

Mori IC and Muto S 1997 Abscisic acid activates a 48-kilodalton protein kinase in guard cell protoplasts. *Plant Physiol.* **113** 833–839

Nürnberger T, Nennstiel D, Jabs T, Sacks WR, Hahlbrock K and Scheel D 1994 High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defense responses. *Cell* **78** 449–460

Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, *et al*. 2009 Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324** 1068–1071

Pei ZM, Murata Y, Benning G, Thomine S, Klüsener B, Allen GJ, Grill E, Schroeder JI, *et al*. 2000 Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* **406** 731–734

Raghavendra AS, Gonugunta VK, Christmann A and Grill E 2010 ABA perception and signaling. *Trends Plant Sci.* **15** 395–401

Reyes D, Rodriguez D, Gonzalez-Garcia MP, Lorenzo O, Nicolás G, García-Martínez JL and Nicolás C 2006 Overexpression of a protein phosphatase 2C from beech seeds in Arabidopsis shows phenotypes related to abscisic acid responses and gibberellin biosynthesis. *Plant Physiol*. **141** 1414–1424

Reymond P, Grunberger S, Paul K, Müller M and Farmer EE 1995 Oligogalacturonide defense signals in plants: Large fragments interact with the plasma membrane in vitro. *Proc. Natl. Acad. Sci. USA* **92** 4145–4149

Saravanakumar D, Vijayakumar C, Kumar N and Samiyappan R 2007 PGPR-induced defense responses in the tea plant against blister blight disease. *Crop Prot.* **26** 556–565

Schmidt C, Schelle I, Liao YJ and Schroeder JI 1995 Strong regulation of slow anion channels and abscisic acid signaling in guard cells by phosphorylation and dephosphorylation events. *Proc. Natl. Acad. Sci. USA* **92** 9535–9539

Sharp JK, Albersheim P, Ossowski P, Pilotti A, Garegg P and Lindberg B 1984 Comparison of the structures and elicitor activities of a synthetic and a mecelial-wall-derivedHexa (β-D-glucopyranosyl)-D-glucitol. *J. Biol. Chem.* **259** 11341–11345

Sheen J 1998 Mutational analysis of protein phosphatase 2C involved in abscisic acid signal transduction in higher plants. *Proc. Natl. Acad. Sci. USA* **95** 975–80

Strobel NE, Ji C, Gopalan S, Kuc JA and He SY 1996 Induction of systemic acquired resistance in cucumber by *Pseudomonas syringae pv. syringae* 61 HrpZ $_{Pss}$ protein. *Plant J.* **9** 431–439

Suzuki N, Miller G, Morales J, Shulaev V, Torres MA and Mittler R 2011 Respiratory burst oxidases: the engines of ROS signaling. *Curr. Opin. Plant Biol.* **14** 691–699

Svalheim Ø and Robertsen B 1993 Elicitation of H_2O_2 production in cucumber hypocotyl segments by oligo-1, 4-α-D-galacturonides and an oligo-β-glucan preparation from cell walls of *Phythophthora megasperma f. sp. Glycinea*. *Physiol. Plant* **88** 675–681

Szostkirwiez I, Richter K, Kepka M, Demmel S, Ma Y, Korte A, Assaad FF and Christmann A 2010 Closely related receptor complexes differ in their ABA selectivity and sensitivity. *Plant J.* **61** 25–35

Tahtiharju S and Palva T 2001 Antisense inhibition of protein phosphatase 2C accelerates cold acclimation in Arabidopsis thaliana. *Plant J*. **26**(4) 461–470

Thuerig B, Felix G, Binder A, Boller T and Tamm L 2006 An extract of *Penicillium chrysogenum* elicits early defense-related responses and induces resistance in *Arabidopsis thaliana* independently of known signalling pathways. *Physiol. Mol. Plant Pathol.* **67**(3) 180–193

Torres MA and Dangl JL 2005 Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. *Curr. Opin. Plant Biol.* **8**(4) 397–403

Torres MA, Dangl JL and Jones JDG 2002 Arabidopsis gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proc. Natl. Acad. Sci. USA* **99** 523–528

Torres MA, Jones JD and Dangl JL 2006 Reactive oxygen species signaling in response to pathogens. *Plant Physiol.* **141** 373–378

Tougane K, Komatsu K, Bhyan SB, Sakata Y, Ishizaki K, Yamato KT, Kohchi T and Takezawa D 2010 Evolutionarily conserved regulatory mechanisms of abscisic acid signaling in land plants: characterization of ABSCISIC ACID INSENSITIVE1-like type 2C protein phosphatase in the liverwort *Marchantia polymorpha*. *Plant Physiol.* **152** 1529–1543

Umezawa T, Sugiyama N, Mizoguchi M, Hayashid S, Myougae F, Yamaguchi-Shinozakic K, Ishihamab Y and Hirayamad T 2009 Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **6** 17588–17593

Vos IA, Verhage A, Schuurink RC, Watt LG, Pieterse CM and Van Wees SC 2013 Onset of herbivore-induced resistance in systemic tissue primed for jasmonate-dependent defenses is activated by abscisic acid. *Front. Plant Sci*. **4**(2) 539

Yoshida T, Nishimura N, Kitahata N, Kuromori T, Ito T, Asami T, Shinozaki K and Hirayama T 2006 ABA-hypersensitive germination3 encodes a protein phosphatase 2C (AtPP2CA) that strongly regulates abscisic acid signaling during germination among Arabidopsis protein phosphatase 2Cs. *Plant Physiol*. **140**(1) 115–126

Yoshioka H, Numata N, Nakajima K, Katou S, Kawakita K, Rowland O, Jones JDG and Doke N 2003 *Nicotiana benthamiana* gp91phox homologs NbrbohA and NbrbohB participate in H_2O_2 accumulation and resistance to *Phytophthora infestans*. *Plant Cell* **15**(3) 706–718

Zhang X, Zhang L, Dong FC, Gao J, Galbraith DW and Song CP 2001 Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in *Vicia faba*. *Plant Physiol*. **126**(4) 1438–1448

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